Xylogenesis and hormones in soybean callus. I: A histological and ultrastructural study

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Five ultrastructural developmental stages are described for differentiating tracheary elements in callus of *Glycine max* (L.) Merr. Xylogenesis was induced by exogenous application of indolebutyric acid (IBA) + kinetin, IBA + *trans*-zeatin, IBA + benzyladenine (BA) and IBA + gibberellic acid (GA_3). Initiation of tracheary elements occurred after division of the callus cells and was followed by cell enlargement, secondary wall deposition and lignification, all of which culminated in the final autolysis of the immature tracheary elements. Mature elements included both tracheids with scalariform to reticulate wall sculpturing and pitted vessels with simple perforations. Nodulation was extensive in all the treatments. A lateral pattern in autolysis of tracheary elements is thought to be a reflection of a concomitant migration of the inductive stimulus ultimately resulting in groups of xylem elements.

Vyf ultrastrukturele ontwikkelingstadia vir differensiërende trageale elemente in *Glycine max* (L.) Merr. (soyaboon) kallus word beskryf. Xilogenese is deur die toediening van indoolbottersuur (IBA) + kinetien, IBA + *trans*-zeatien, IBA + bensieladenien (BA) en IBA + gibberelliensuur (GA_3) geïnduseer. Trageale elemente is na seldeling geïnisieer en is gevolg deur selvergroting, sekondêre selwand-deposisie en lignifikasie, wat uiteindelik tot outolise van die ontwikkelende elemente lei. Volwasse elemente is gekenmerk deur beide trageïede met leervormige tot netvormige selwandstrukture en stippelhoutvate met eenvoudige perforasies. Bondelvorming is volop in al die behandelings. 'n Laterale outolisepatroon in trageale elemente word toegeskryf aan 'n gepaardgaande migrasie van die induktiewe stimulus om uiteindelik in groepies xileemelemente te eindig.

Keywords: Hormones, nodulation, tracheary elements, ultrastructure

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Introduction

Ultrastructural features of developing primary xylem elements in intact plants such as *Phaseolus vulgaris* L. (Esau & Charvat 1978) are fairly well documented. A general ontogenetic pattern ranging from the development of an induced parenchyma cell to a mature xylem element with secondary cell wall thickening and the presence of an occasional perforation plate has been described (Esau 1977; O'Brien 1981).

Although the effects of hormonal supplements on xylogenesis in cultured plant material have been quantified in many systems (Roberts 1976; Shininger 1979; Minocha 1984), very little information exists on the histology of the induced xylem elements in the tissues investigated. *In vitro* induced xylogenesis in most species produced both vessels and tracheids with scalariform to reticulate wall thickenings. Giant tracheids occasionally occurred in some gibberellic acid treatments (Cawthon 1972; Snijman 1972). Tracheary elements are usually arranged to form clusters or nodules and these are reported to be accompanied by phloem (Abbott 1974; Aloni 1980). A cyto-differential sequence of development under *in vitro* conditions can be outlined as follows: cell division (Comer 1978; Tucker *et al.* 1986), enlargement, secondary wall hydrolysis and cell degradation (Roberts 1976).

This paper describes the development of hormonally induced tracheary elements in an *in vitro* soybean callus system.

Materials and Methods

Plant material

Glycine max (L.) Merr. var. Acme callus, maintained on a Miller's (1965) growth medium supplemented with 10^{-9} mol dm^{-3} napthalene acetic acid (NAA) and $0{,}5 \times 10^{-7}$ mol dm^{-3} kinetin, served as a stock callus for experiments. The stock culture which was grown at ± 25°C and low light intensity (0,5 μl m^{-2} s^{-1}), was subcultured every 2 months. For the experimental studies callus was grown on a Murashige & Skoog (1962) basal medium supplemented with 100 mg l^{-1} myo-inositol, 3% sucrose and various hormone combinations. Treatments which, after quantitative analysis of macerated tissue, yielded the greatest number of tracheary elements for each hormone combination were examined microscopically. These treatments were: no hormones (control); $0{,}5 \times 10^{-4}$ mol dm^{-3} indolebutyric acid (IBA) + 10^{-5} mol dm^{-3} kinetin; $0{,}5 \times 10^{-4}$ mol dm^{-3} IBA + 10^{-6} mol dm^{-3} benzyladenine (BA); 10^{-5} mol dm^{-3} IBA + 10^{-6} *trans*-zeatin and 10^{-5} mol dm^{-3} IBA + 10^{-7} mol dm^{-3} gibberellic acid (GA_3).

Microscopy

The plant material was harvested after 7, 14 and 21 days and treated in two ways. The callus tissue was fixed in formalin–alcohol–acetic acid (FAA) whereafter it was dehydrated in a tertiary butanol series (Johansen 1940) and infiltrated with wax. Semi-thin (10 μm) sections were cut on a Jung rotary microtome and stained with safranin (Johansen 1940). Alternatively, at the termination of the experiment, the plant material was fixed in 3% glutaraldehyde buffered with 0,05 mol dm^{-3} sodium cacodylate (pH 7,2). Normal electron microscopic procedures were subsequently followed, these being post-fixation in 2% osmium tetroxide, alcohol dehydration, infiltration and embedding in Epon araldite resin. Ultrathin sections (0,05 μm) were sectioned on a Reichert Jung Ultracut E ultramicrotome and examined with a JEOL 100 CX electronmicroscope at an accelerating voltage of 80 kV. When required maceration of the callus was done in a 1:1 solution of HCl:chromic acid for 24 h at 25°C.

Results and Discussion

Xylem elements were grouped into very definite nodules and occurred throughout the callus regardless of the treatment.

The nodules were of two types and were similar to those previously described for *Nicotiana tabacum* L. (Abbott 1974). Nodulation in all the IBA + kinetin and IBA + GA_3-treated tissues were of the type where xylem and phloem were adjacently arranged (Figure 2). In the second type of arrangement xylem elements were surrounded by crushed phloem elements. This was only found in IBA + BA treatments.

The tracheary elements formed were of various shapes and sizes in soybean callus. Roberts (1976) reported that such development occurred with all hormonally induced tracheary elements in *in vitro* systems. Both vessels and tracheids were observed in soybean callus with tracheids being more common. Wall sculpturing varied from reticulate to pitted (Figure 1). Tracheary elements with reticulate walls and simple perforations were found in *Coleus blumei* Benth. (Comer 1978) while Liskova (1985) described pitted elements in *Picea excelsa* Link callus. Vessel members in soybean had simple perforations and pitted wall sculpturing (Figure 3). Pit membranes (Figures 3 & 4a), which usually originate from hydrolysed primary walls (Gunning & Steer 1975), underlie the simple pits which were often adorned with wartlike excrescences (Figure 3). These excrescences are possibly cytoplasmic remnants which arose during maturation of the element (Gunning & Steer 1975). Absence of the pit membranes and excrescences could be due to preparation methods.

Large thin-walled parenchyma cells which gave a positive stain for lignin were found in callus treated with 10^{-5} mol dm^{-3} IBA + 10^{-5} mol dm^{-3} GA_3 (Figure 5). Similar cells were previously recorded in *Nicotiana* (Snijman 1972) and *Lactuca sativa* L. (Cawthon 1972) pith explants. It was suggested that gibberellins may have an effect on microtubule arrangement and thus affect cell wall depositioning

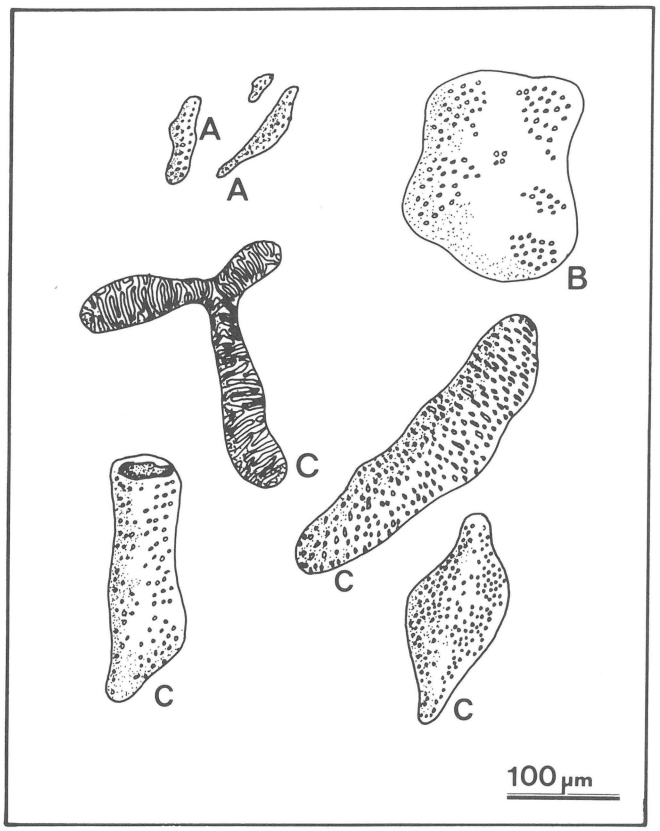

Figure 1 An illustration of tracheary elements obtained from macerated soybean callus. A = no hormones; B = IBA + GA_3; C = elements common to all the treatments.

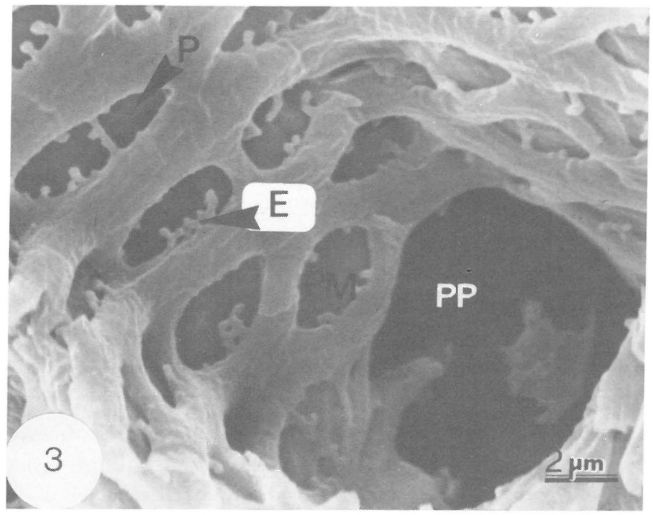

Figure 3 Scanning electron micrograph showing a vessel with simple pits (P) adorned with wall excrescences (E) and simple perforation (PP). PM = pit membrane.

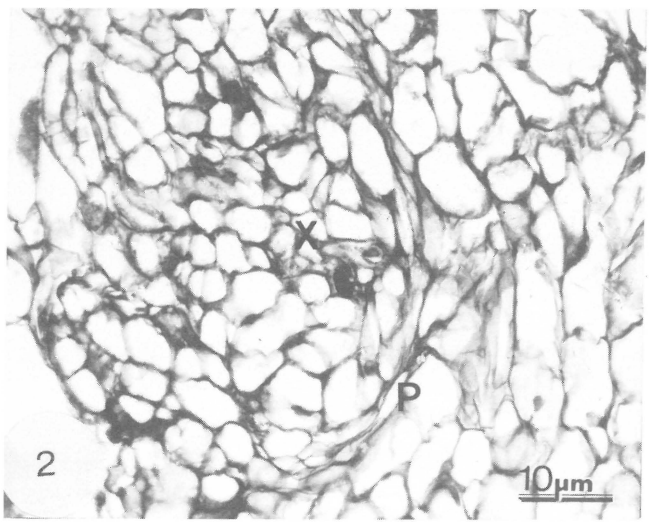

Figure 2 Vascular nodules in soybean callus treated with IBA + kinetin. P = phloem; X = xylem.

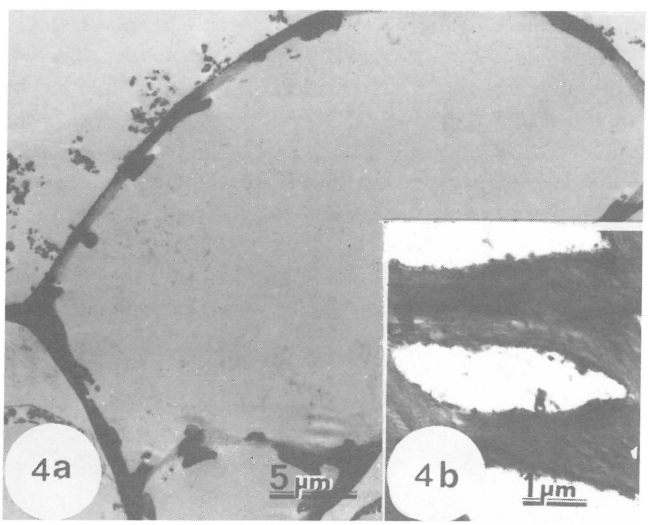

Figure 4 Transection showing part of an induced tracheid (a) with reticulate wall sculpturing (b).

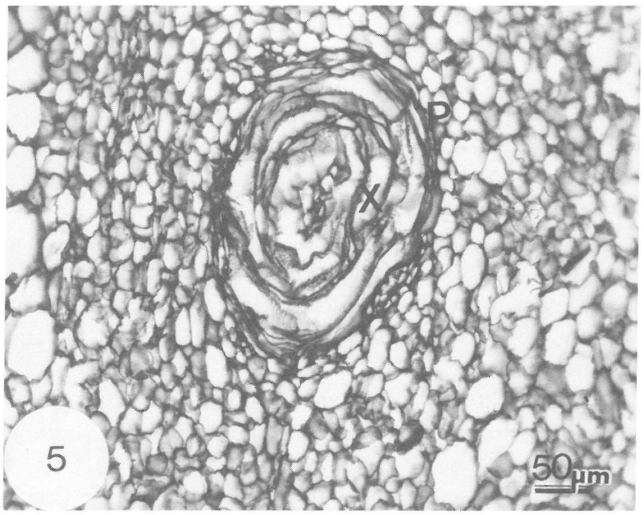

Figure 5 Vascular nodule in soybean callus treated with 10^{-5} mol dm^{-3} IBA + 10^{-5} mol dm^{-3} GA_3. P = phloem; X = xylem.

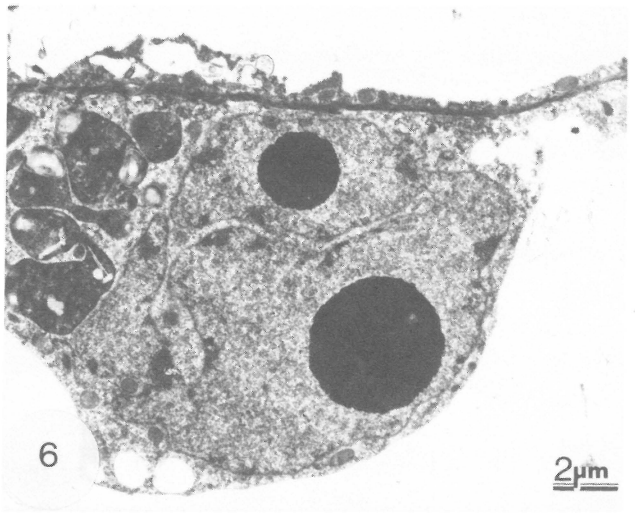

Figure 6 Electron micrograph showing a multinucleolate cell (or highly branched nucleus) typical of IBA + *trans*-zeatin treatments in induced tracheary elements.

(Torrey *et al.* 1971).

Development of these cells could very likely be due to a dual effect of auxins and gibberellins on expansion and longitudinal cell growth. Longitudinal growth was promoted by the orientation of microtubules in coleoptiles. Callus cultured in the absence of hormones were generally less responsive.

Five developmental stages (phases) were recorded for the development of soybean xylem elements; these being division, enlargement, secondary cell wall depositioning, lignification and autolysis.

The developing tracheary elements were distinguished from the surrounding parenchyma by an increase in cell size comprising of both lateral and longitudinal growth. The cytoplasmic arrangement in the immature elements was mainly parietal, but the cytoplasmic density differed the least in IBA + kinetin and the most in the IBA + BA treatments. More than one vacuole was often found in IBA + *trans*-zeatin treatments. Immature tracheary elements of *Phaseolus vulgaris* were characterized by definite parietal vacuolation (Esau & Charvat 1978) while the initial stages of differentiation in *Avena sativa* L. (Cronshaw & Bouck 1965) and *Zea mays* L. (Srivistava & Singh 1972) were marked by extensive vacuolation. During this growth period nuclei of the developing soybean cells became either lobed and enlarged or the cell became multinucleolate (Figure 6) as is found in other systems (Cronshaw & Bouck 1965; Esau 1977; O'Brien 1981).

The ontogeny of a tracheary element in soybean callus did not differ between any of the treatments. The developmental sequence described above was similar for all the treatments.

The fully expanded immature tracheary elements contained all the usual cytoplasmic inclusions. Mitochondria, rough ER and dictyosomes were most frequently arranged adjacent to the plasma membrane (Figure 7). Plastids (amyloplasts) ribosomes and vacuoles were found together with some mitochondria and ER throughout the remainder of the cytoplasm (Figure 8). Microtubules were at first arranged in close proximity to the primary wall (Figure 7) and later concentrated next to the secondary wall areas (Figure 9). At times it seemed fused to the plasmamembrane.

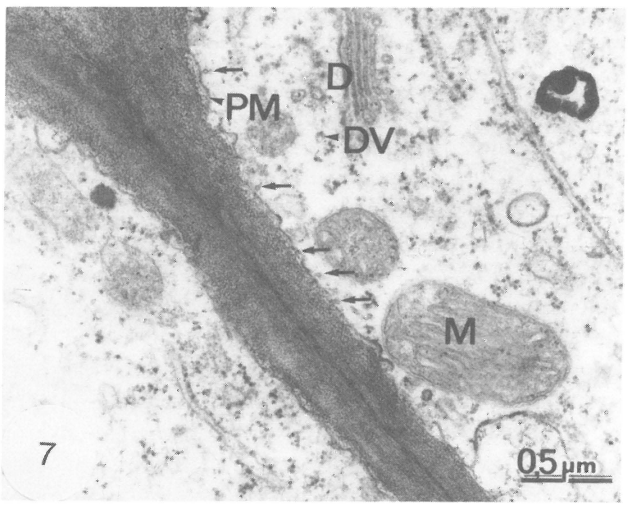

Figure 7 Electron micrograph showing microtubules (→) adjacent to the plasmalemma membrane prior to the onset of secondary wall deposition. D = dictyosome; DV = dictyosome vesicle; M = mitochondrion.

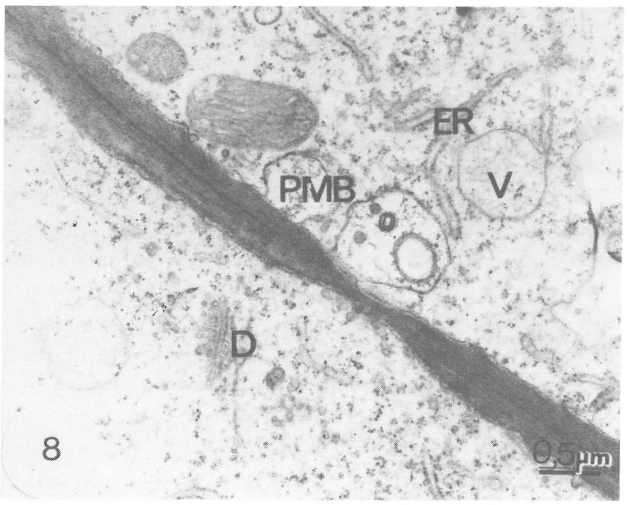

Figure 8 Paramural bodies (PMB) and lytic vacuoles (V) characterize the initial stages of autophagial autolysis. D = dictyosome; ER = endoplasmic reticulum.

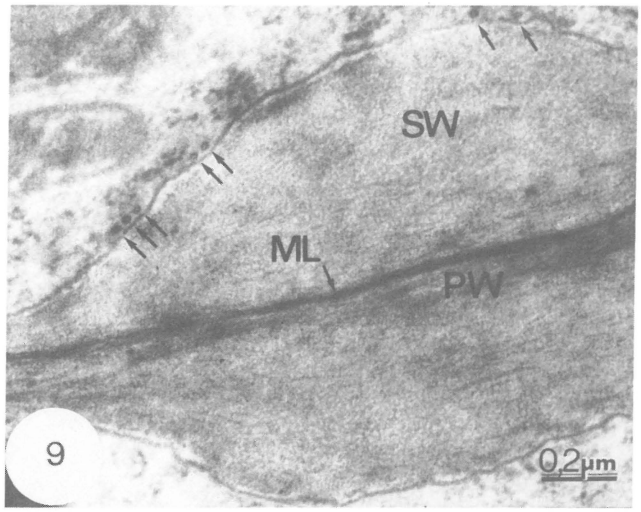

Figure 9 Electron micrograph showing microtubules (→) arranged in close proximity to the secondary wall ridges (SW). ML = middle lamella; PW = primary wall.

Srivistava & Singh (1972) thought that microtubules arranged newly formed microfibrils during wall depositioning. Goosen de Roo (1973) however, proposed that dictyosome vesicles carried wall material to those areas which were free of microtubules. It is questionable whether the site of secondary wall formation is determined by microtubules as they occurred not only along the secondary walls, but also adjacent to the primary walls in both the soybean callus system and *Phaseolus* (Esau & Charvat 1978).

ER occurred mostly in the primary wall areas as well as adjacent to newly deposited secondary wall areas. It was, however, not concentrated between secondary wall ridges as was previously described for *Zea* (Srivistava & Singh 1972). The tonoplast often showed invaginations, and vacuoles (Figure 8) were present in the cytoplasm. These showed membranous inclusions — a possible indication of autophagic activity prior to secondary wall depositioning. Protoplasmic material is possibly recycled during this period.

Paramural bodies occurred adjacent to the primary wall (Figure 8). These are thought to be derived from dictyosome vesicles incorporated into the plasma-membrane during cell wall depositioning (Gunning & Steer 1975; Esau & Charvat 1978). Dictyosomes have most commonly been implicated in cell wall depositioning by means of vesicles (Cronshaw & Bouck 1965; O'Brien 1981). In experiments where labelled glucose was fed to *Marchantia berteroana* L., Fowke & Pickett-Heaps (1972) detected the label firstly in the dictyosomes and later in the cell wall.

Many vesicles were found in close proximity to invaginations of the plasmalemma during the late stages of primary and early stages of secondary wall depositioning (Figure 10). These vesicles are thought to be derived from both ER and dictyosomes. Tanchak *et al.* (1984) described both smooth and coated vesicles (originating from ER and dictyosomes), which were involved in active transport of material of a similar nature in soybean suspension cells. Coated vesicles (Figure 10) were autophageous in nature and appear to be active in endocytosis. Such coated vesicles were present in the earlier stages of tracheary element development in the soybean callus system and could be indicative of the uptake of required stimuli for differentiation.

Cronshaw & Bouck (1965) and Srivistava & Singh (1972) described two types of dictyosome-derived vesicles. The first was electron transparent and thought to deposit hemicellulose pectins and polyurinoids while the second type (electron dense) is possibly involved in lignification as it occurred in the late stages of secondary wall depositioning (Srivistava & Singh 1972). Vesicles were found to be present throughout all the stages of wall depositioning in soybean elements thus suggesting active transport of material. The origin of these vesicles could not be determined but they were associated with both the ER and the dictyosomes.

The secondary cell wall was arranged in discontinuous ridges upon the primary cell wall (Figures 9 & 11). Initially the primary cell wall is uneven, but according to Esau & Charvat (1978) secondary cell wall depositioning occurs once the primary wall and middle lamellae are arranged into even layers. Srivistava & Singh (1972) linked the fibrillar nature of the secondary wall to inclusions of tubular

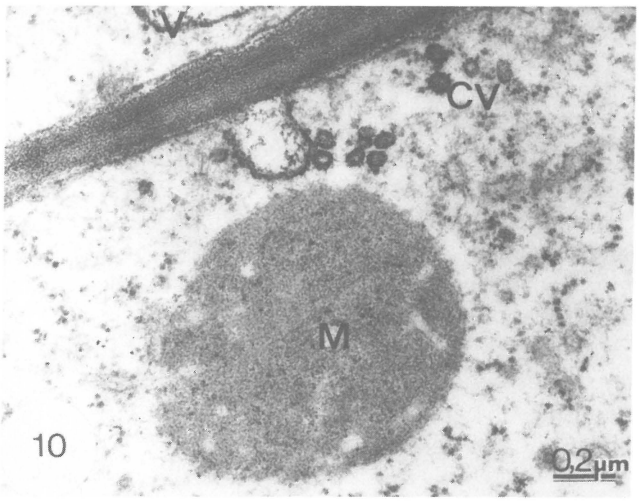

Figure 10 Illustrates coated vesicles (CV) in close proximity to an invagination of the plasmalemma. There is a marked similarity in the nature of the vesicle and plasmalemma. M = mitochondrion; V = vacuole.

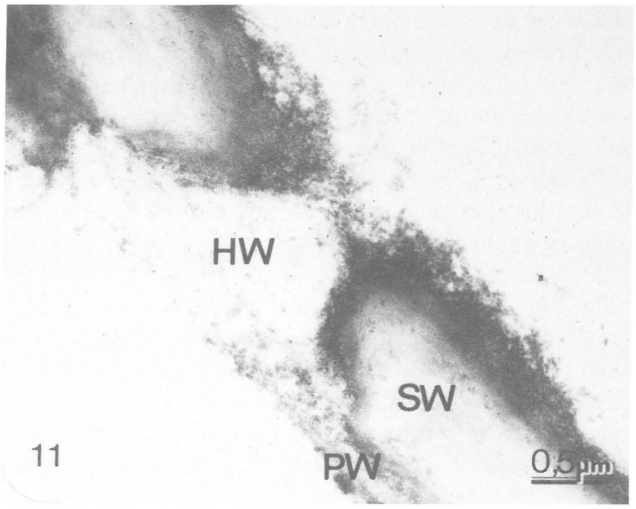

Figure 11 Shows part of the lignified secondary wall (SW) and a hydrolysed primary wall (HW) in the region of the primary pit. PW = primary wall.

ER lamellae. Once secondary cell wall depositioning and subsequent lignification was completed, final sequestration of the cytoplasm commenced (Figure 11).

The first stages of autolysis were marked by the presence of many lytic structures. Cytolysosomes (Figure 12) i.e. concentric arrangements of ER which enclosed portions of cytoplasm (Villiers 1972) developed during this stage. Cytolysosomes are characteristic of intense autophagy (Matile 1975) where the cytoplasm lying within the envelope is rapidly suquestrated by acid hydrolases released from the ER (Cresti *et al.* 1972: Matile 1975).

Furthermore, cytolysosomal activity has been shown to be prominent in cells which show extensive vacuolation during development e.g. development of xylem vessels (Berjak 1973). Paramural bodies are in abundance at this stage (Figure 12). The tonoplast is subsequently ruptured and is said to set highly selective hydrolytic enzymes free (Matile 1975; Esau & Charvat 1978; O'Brien 1981). Hence many vacuolar areas devoid of membranes arise in the cytoplasm. All the cytoplasmic contents including the nucleus degraded — the mitochondria persisted until last. The primary wall not covered by the lignified secondary wall was hydrolysed and a pit membrane consisting of loosely arranged cellulose fibrils (Gunning & Steer 1975) remained. Absence of a pit membrane is probably due to preparation methods. Development of a perforation plate was not observed. All the xylogenetic processes which occurred were thought to be hormonally controlled (Roberts 1976; Esau 1977).

The end result was a mature non-living tracheary element which was often flanked by a parenchymatous cell or immature tracheary element and a mature element. The adjacent parenchymatous cell displayed increased cellular activity with an abundance of ER, mitochondria, dictyosomes and ribosomes which were arranged adjacent to the cell wall. A similar progression in tracheary element development has been described for *Phaseolus* (Esau & Charvat 1978). Roberts (1976) ascribed such a trend to the release by autolysing tracheary elements of a stimulus which then migrates from one cell to another. There is also the possibility that a vertical system, via perforations, rather than a lateral system could be involved in xylem induction in some systems. A lateral system appears to be favoured by the soybean callus. Optimal concentrations of the required stimulus, which is possibly mimicked by exogenously applied hormones, would decrease in activity after a time period thus explaining limited areas of nodulation.

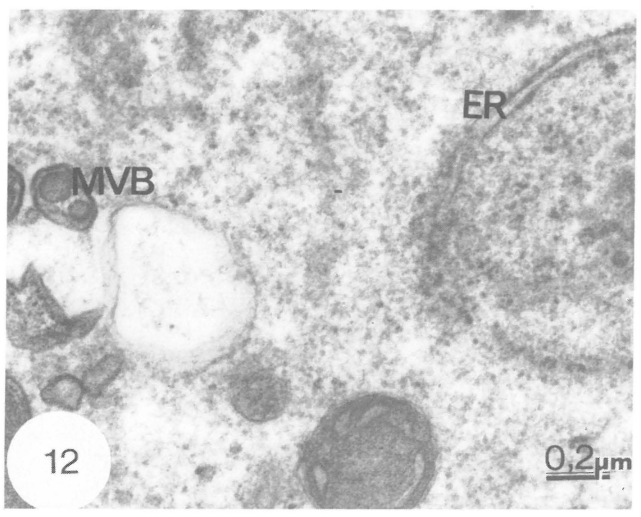

Figure 12 Cytolysosomes, delimited by endoplasmic reticulum (ER), are found in autolysing tracheary elements. MVB = multivesicular body.

Acknowledgements

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